

Combination of Doxorubicin and Areca Ethanolic Extract Induces Apoptosis by Increasing Caspase-3 Level on Breast Cancer (T47D) Cells

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Abstract

Despite causing many side effects, doxorubicin (Dox) is still one of breast cancer drug of choice. Thus, combination of chemotherapy is developed in order to decrease doxorubicin regimen dose. The aim of this research is to examine the combination effect of doxorubicin (dox) and areca extract (AE) on T47D human breast cancer cells. The cytotoxic activity was determined using MTT assay. The combination index (CI) of the combination treatment was calculated to determine the effects (synergistic, additive or antagonistic). The combination application of dox (6-22nM) and AE (8-30µg/ml) on T47D cells showed synergistic (CI<0.9) or additive effect (CI=0.9-1.1). The effective combination of dox-AE was 6 nM - 8 µg/mL on CI<0.5. Apoptosis induction of AE solely and its combination with dox was then observed using double staining method. Moreover, expression of Bax and caspase-3 protein which mediated apoptosis, were observed using immunocytochemistry. Combination of AE and Dox increased expression of Caspase-3 but did not increase expression of Bax. This result showed AE increase the effectiveness of doxorubicin against T47D cells.

Keywords: Breast cancer, doxorubicin, areca extract, T47D cells

INTRODUCTION

Chemotherapy is the most effective therapy for all stages of breast cancers. Doxorubicin is a chemotherapeutic agent which is widely used for breast cancers (Smith, *et al.*, 2006). Doxorubicin interacts with DNA by covalent binding thus inhibit DNA synthesis and RNA-polymerase activity (Mullin and Claude, 1998). Beside, doxorubicin generates reactive oxygen species (ROS) which initiate series of reaction disturbing cell integrity (Fang and Iyer, 2007). Long term use of doxorubicin induces hepatotoxicity and haematopoietic imbalance (Arai, *et al.*, 2000; Jemal, *et al.*, 2005), and resistance mediated by P-glycoprotein (Pgp) overexpression. Pgp effluxes drug from the cell and reduces drug intracellular (Thomas and Coley, 2003).

Pinang seed (*Areca catechu* L.) is used for traditional food spice. Meiyanto, *et al.* (2008) reported that ethanolic extract of pinang seed inhibit MCF-7 breast cancer cells proliferation and induces apoptosis. MCF-7 is p53 expressing cells. Ethanolic extract of

pinang seed also showed antioxidant activity with IC₅₀ value of 45.5 µg/mL (Wetwitayaklung, *et al.*, 2006). Antioxidant activity is usually correlated with cancer prevention. Thus, a combination of chemotherapeutic agent and chemopreventive agent which has been known to increase anticancer activity and decrease side effects, is an effective strategy to cure cancer (Wetwitayaklung, *et al.*, 2006).

Normally, apoptosis is occurred to achieve homeostatic condition. Nevertheless, cancer cells which are genetically modified have ability to avoid apoptosis (Hanahan and Weinberg, 2011). This research was conducted to observe combination effect of doxorubicin (Dox) and areca extract (AE) on cell cytotoxicity, induction of apoptosis, and mechanism mediated apoptosis on T47D breast cancer cells.

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MATERIALS AND METHODS

Materials

Ethanollic extract of pinang seed/areca extract used in this research was prepared and standardized based on Meiyanto, *et al.* (2008). T47D cells were a collection of CCRC (*Cancer Chemoprevention Research Center*) Faculty of Pharmacy UGM which is originally obtained from Laboratory of gene function, NAIST, Japan. Cells were grown in medium cultur DMEM (*Dulbecco's modified Eagle's Medium*) contains FBS (*Fetal Bovine Serum*) 10% and penicillin-streptomycin 1% (Gibco). Tripsin-EDTA was used to dettached cells on the flask.

Cytotoxic Assay

Cytotoxic assay of Dox and AE alone against T47D cells were conducted to determine concentration used in combination cytotoxic assay. Cytotoxic assay was done using MTT, as validated by Itagaki, *et al.* (1998). T47D cells (3×10^3 cells/well) were suspended in 100 μ L of culture medium. Dox (nM) and AE (μ g/mL) concentration used in the combination assay are 22-30, 11-30, 22-15, 11-15, 6-15, 11-8, 6-8. Combination index was calculated based on equation from Notarbartolo, *et al.* (2005).

Apoptosis Detection

Apoptosis was detected using acrydine orange-ethidium bromide staining (AO/EB double staining). T47D cells (3×10^4 cells/well) were seeded on coverslips in 24- well plates until 50-60% confluent. Cells were then incubated with AE alone, Doxorubicin alone or their combination for 48 h. Culture medium was removed and cells were washed with PBS. Coverslips were placed into object-glass and added with 10 μ L of 1x working solution acrydine orange (Sigma)-ethidium bromide (Sigma), observed using fluorescence microscopy (Zeiss MC 80). Apoptotic cells which had lost their membrane integrity appeared orange and showed morphological features of apoptosis. Cells were identified as apoptotic cells based on the specific morphological criteria, including condensation

and fragmentation of chromatin, and formation of apoptotic bodies.

Immunocytochemistry

Cells were plated at 3×10^4 cells/well and cultured in 24-wells plate at cover slip until 80 % confluent. When the cells were ready to be treated, medium was replaced by fresh complete medium with AE 24 μ M and doxorubicin 500 nM and incubated in CO₂ 5% incubator at 37°C for 18 hours. Then, cells were harvested and were washed with PBS and fixed with cold methanol for 10 min at freezer -40C. Cells were washed, and blocked in hydrogen peroxide blocking solution for 10 minutes at room temperature. After that, cells were washed with PBS and incubated with prediluted blocking serum for 10 minutes at room temperature. Cells were stained for 1 h at room temperature with primary Ab (caspase-3 and Bax). After being washed three times in PBS, secondary antibody were applied for 15-30 min, 1 : 2 in PBS and added with 5% AB serum then washed with PBS for three times. The slide was incubated with streptavidin-biotin complex for 15 min, 1 : 2 in PBS and added 5% AB serum and washed three times in PBS. Slides were incubated in DAB (3,3diaminobenzidin) solution for 3-8 min and washed with aquadest. Cells were counterstained for 3-4 min with Mayer-Haematoxylin. After incubation, coverslip were washed with aquadest and immersed. Protein expression was assessed under light microscope. Positive expression will give a dark brown colour in nucleus and cells with no expression will give purple colour.

RESULTS AND DISCUSSION

Effect of Areca Extract (AE) and Doxorubicin Solely on T47D Cells Viability

Doxorubicin showed cytotoxic effect on T47D cells in dose dependent manner (Fig.1(A)). At the concentration of 32 nm, dox inhibited 50% of cell viability. AE also showed cytotoxic effect on T47D cells (Fig.1(B)). Based on linier regression between log of concentration and cell viability percentage ($p < 0.05$), IC₅₀ value of AE is 50 μ g/mL.

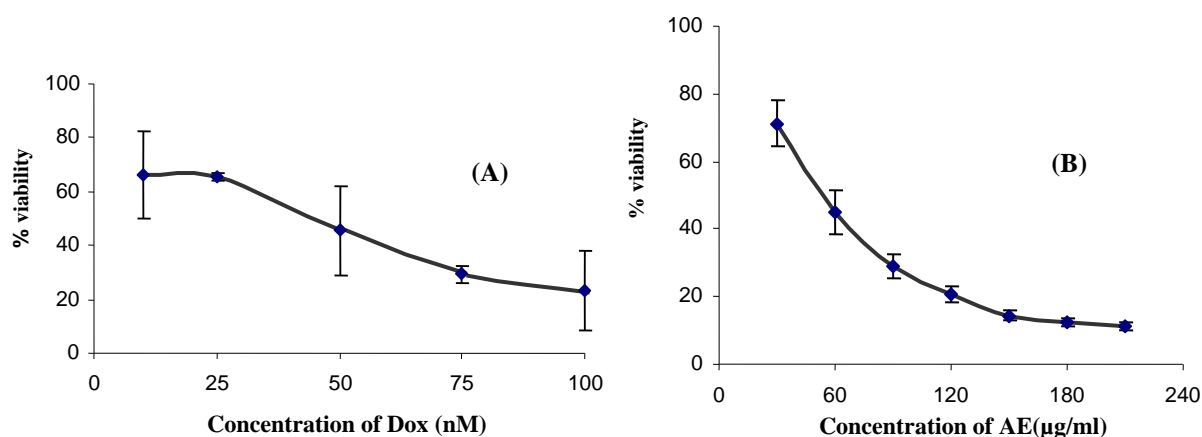


Figure 1. Cytotoxic effect of Doxorubicin and Ethanolic extract of Areca (AE) on T47D cells. IC_{50} values obtained from the calculation of linear regression of concentration vs % viable cells with $p < 0.05$. (A) doxorubicin (10-100 nM) and (B) AE (30-210 µg/ml). Cell viability were obtained from the conversion of absorbance values of formazan that was formed by MTT treatment as described in the method. AE and Dox cytotoxicity expressed by percent cell viability is shown as the mean + SE of 3 experiments.

Effect of Combination of AE and Doxorubicin on T47D Cells Growth

Combination index at combination of doxorubicin and AE is presented in Table I. Almost all of combination gave synergistic

effect ($CI < 0.9$). However, dox 22 nM combined with AE 15 µg/ml gave additive effect (CI 0.9-1.1). Based on these results, combination treatment showed increase cytotoxicity of doxorubicin.

Table I. Combination index value given by combination of dox and AE

EP (µg/ml)	Dox (nM)		
	22	11	6
30	0,86	0,79	-
15	0,96	0,66	0,60
8	-	0,66	0,35

Effect of Combination of AE and Doxorubicin on Apoptosis

Apoptosis induction on T47D cells was observed after 48 hours of incubation. Cells were treated with AE (60 µg/mL), doxorubicin (44 nM), or combination of them. AE and dox

induced T47D cells apoptosis for 5% and 6%, respectively (Fig.2). Combination of AE and dox increase apoptosis induction (23%) compared to each compound. This result showed synergistic effect between dox and AE.

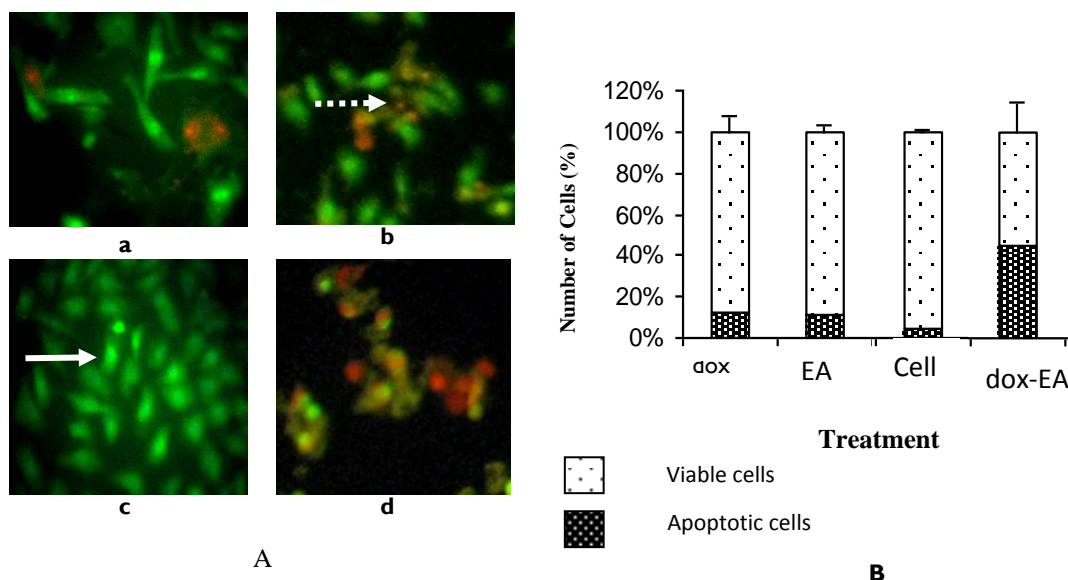


Figure 2. Effect of AE and Dox on T47D cells apoptosis using acridine orange-ethidium bromide staining. (A) Cell morphology after treated with (a) doxorubicin 44 nM (b) AE 60 µg/mL (c) untreated (d) combination of dox-AE. (B) Apoptosis quantification graphic. Apoptotic cell (···▶) and viable cell (—▶)

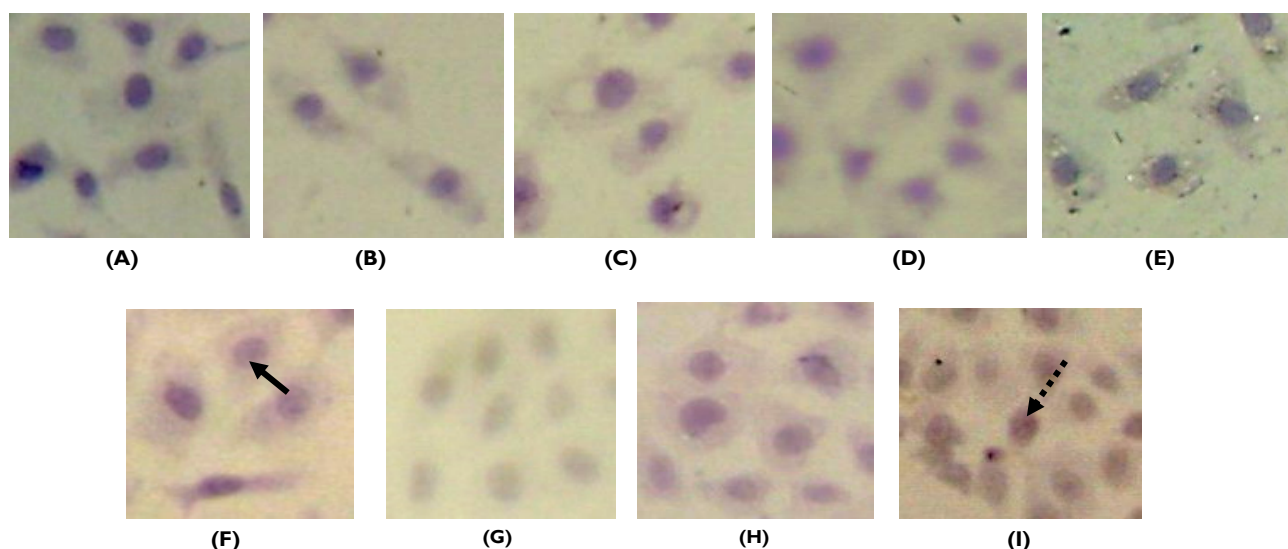


Figure 3. Effect of AE and Dox on Bax and Caspase 3 expression in T47D cells. Bax and caspase 3 expression was observed using immunocytochemistry as described in method and treated with (A) cells without Bax antibody (B) control with Bax-Ab (C) dox 70 nM with Bax-Ab (D) AE 70 µg/mL with Bax-Ab (E) combination of Dox-AE with Bax-Ab (F) control with caspase-3-Ab (G) dox 70 nM with caspase-3-Ab (H) AE 70 µg/mL with caspase-3-Ab (I) combination of Dox-AE with caspase-3-Ab. Cells were observed under light microscope with 100x magnification. cells expressed caspase-3 (—▶) cells did not expressed caspase-3 (···▶)

Effect of Combination of AE and Doxorubicin on Bax and Caspase-3 Expression

To confirm the mechanism of AE and its combination with doxorubicin induced apoptosis, we observed the effect of AE, doxorubicin or their combination on the expression of Bax, and caspase-3 by using immunocytochemistry method. However, AE and Dox did not increase Bax level (Fig. 3(B-E)). On the other hand, all treatment increased caspase-3 level, especially on the combination treatment (Fig.3 (F-I)). This result indicates there are increase of caspase-3 level by all treatment, especially by combination treatment. These data showed that the combination was more potent to induce apoptosis on the T47D cells than single treatment.

Generally assumed that plant extract with $IC_{50} < 100 \mu\text{g/ml}$ is potential to be developed as anticancer agent (Ueda, *et al.*, 2002). Accordingly, the results of this study demonstrated potency of areca extract (AE) to be developed as anticancer agent, showed by its IC_{50} on T47D cells. Doxorubicin as drug of choice for breast cancer has been related with resistance phenomenon in time and dose dependent manner. Moreover, AE showed synergistic effect when combined with doxorubicin, demonstrated its potency to reduce doxorubicin resistance in cancer cells.

Cytotoxic properties can be associated with the ability to spur occurrence of cell cycle arrest and/or accelerate apoptosis on cells. We found that AE and its combination with doxorubicin induced apoptosis on T47D cells. There are two mechanisms mediating apoptosis: extrinsic pathway via death receptor and intrinsic pathway via mitochondria. Some proteins involved in apoptosis, such as Bax. Bax translocation from cytosol to mitochondria caused apoptosis on staurosporine treated T47D cells (Mooney, *et al.*, 2002). Bax must form a dimer to enter mitochondria (Korsmeyer, *et al.*, 2000). In the cells without apoptosis signal, Bax binds to Bcl-2, an antiapoptotic protein. Bcl-2 overexpression lead to apoptosis resistance. In contrast, decrease of Bcl-2 level caused increasing of dimer Bax leading to apoptosis. Beside Bax, caspase-3 activation also involved in apoptosis. Caspase-3 is an effector protein to initiate cell degradation. Apoptosis occurred in staurosporine induced T47D cells was estimated through caspase-3

activation. Therefore, we observed caspase-3 status on T47D cells treated with AE and Dox.

Our study showed that AE and its combination with doxorubicin increased cleaved caspase-3 level but not Bax level. Bax expression is affected by its upstream protein, p53. In T47D cells, p53 protein has been mutated, thus apoptosis induction caused by combination of Dox-AE may not be mediated through p53 pathways. Further mechanism related with apoptosis induction is needed to be observed, such as increase the activation of caspase-3 or related with apoptosis extrinsic pathways. Overall, AE increases the effectiveness of doxorubicin. Nevertheless, this result needs further research to explore the potential of doxorubicin combined with Areca, especially the molecular mechanisms facilitated in increasing effect of doxorubicin.

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